The Effect of Butyrate in the Healing of Colonic Anastomoses in Rats.

CERTIFICATE

This is to certify that the thesis entitled “The Effect of Butyrate on the Healing of Colonic Anastomoses in Rats” is based on the work carried out by Dr. Arpit Jacob Mathew in partial fulfilment of the requirements for MS (Branch I) General Surgery examination of the Tamil Nadu Dr. MGR Medical University to be held in March 2007.

The candidate has independently reviewed the literature and carried out the techniques towards completion of the thesis. This thesis has not been submitted for the award of any degree or diploma of any other University.

Dr Aravindan Nair
Professor and Head
Department of Surgery, Unit VI.
Christian Medical College,
Vellore 632004.

Dr V. Sitaram
Professor and Head
Department of General Surgery
Christian Medical College,
Vellore. 632 004.

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The Effect of Butyrate in the Healing of Colonic Anastomoses in Rats.

A thesis submitted to the Tamil Nadu Dr. M.G.R. Medical University in partial fulfillment of the degree MS (Branch 1) Gen. Surgery

By Arpit Jacob Mathew, Christian Medical College, Vellore, Tamil Nadu. 632004. India

1.0 Introduction

Throughout medical history there have been efforts to develop an ideal method of performing intestinal anastomoses. Over a century ago Nicholas Senn of Chicago, in his presidential address to the Association of Military Surgeons in 1893, said 'We have reasons to believe that the technique of intestinal suturing remains an unfinished chapter and the ideal method of uniting intestinal wounds has yet to be devised'[1]. That statement holds true even today, more than a century later. Wound healing that occurs at colonic anastomoses is multifactorial and disturbed healing results in leakage or rupture with associated morbidity. Low colonic anastomoses have clinically significant leakage rates of around 16%[2] with radiologically demonstrable ones ranging from 27 – 49%[3,4]. Clinical anastomotic leaks necessitate a diversion stoma often prophylactically[5]. The healing of colonic anastomoses is a subject of much debate and research but the factors involved are as yet imperfectly understood[3]. This study proposes to look at the role of one such intraluminal factor in the healing of colonic anastomoses with possible clinical ramifications.

The healing of colonic anastomoses is a subject of great interest to surgeons due to the high rates of morbidity associated with them even today. There have been many attempts to reduce the rate of

complications with different authors suggesting various modifications in the technical construction of these anastomoses, the use of protective stomas, preoperative preparation, early identification of leaks and post-operative management. However, the factors contributing to the healing of these anastomoses are many and there is still much research required to standardise the process and minimise morbidity.

An area which has not yet been fully understood is the physiological basis of colonic healing and the factors that promote and inhibit it. While the physiology of colonic healing has been described, there has not yet been much work attempting to identify treatment options that would encourage early colonic healing at a molecular level. This study was carried out in an attempt to detect a modifiable factor involved in wound healing that could be manipulated to hasten colonic healing following anastomosis.

The premise of the study was that butyrate strengthens colonic anastomoses. This was assumed considering previous work done using butyrate. It has been proved that stimulation of the lumen by even non-nutrient substances provided strong trophic stimuli to the colon.[6] Even infusion of an electrolyte solution enhanced colonic healing in comparison to complete diversion proving that mechanical, non-nutrient stimulation of the enterocytes promotes healing. The physiological production of butyrate in the colon and its role in

providing upto 80% of colonocyte energy made it a natural choice as a factor that would assist colonic healing.

Considering the weight of evidence suggesting that butyrate played a role in cytoprotection and wound healing, this study was carried out to investigate that role and look at the factors that may cause this effect.

2.0 Aims and Objectives

1. To assess the effect of intraluminal butyrate on the healing of anastomoses in rat colon by testing mechanical strength.

2. To compare the differences in the mechanical strength of ascending and descending colon with and without treatment with butyrate.

3. To assess the expression of Matrix Metalloproteinases in the healing colon and study the effect of butyrate on this expression.

3.0 Review of Literature

3.1 Background

The body's ability to replace injured or dead cells and repair tissues following inflammation is critical to survival. The repair of tissues involves two distinct processes – replacement of injured or dead cells by cells of the same type or *regeneration* and replacement by connective tissue called *fibroplasia* or *fibrosis.* Both these processes are determined by essentially similar mechanisms involving cell migration, proliferation and differentiation as well as cell-matrix interactions. The orderly regeneration of epithelial tissue requires a specialised extracellular matrix called the basement membrane which acts as a scaffolding. These processes are the basis of the complex phenomenon of wound healing.

3.2 Regeneration

Cell proliferation is stimulated by cell injury, death or mechanical deformation of tissues. The cell growth cycle consists of the G_1 (presynthetic), S (DNA synthesis), G_2 (premitotic and M (mitotic) phases. Most tissues of the body contain a combination of continuously

dividing cells or *labile* cells, *quiescent* or stable cells that occasionally enter into the cell cycle and *nondividing* cells. Cell replication is controlled mainly by the factors in the microenvironment which stimulate or inhibit cell proliferation. The most important factors are those that recruit resting or quiescent cells into the cell cycle.[7]

The columnar epithelium of the gastrointestinal tract contains a greater proportion of labile cells that follow the cell cycle from one mitosis to the next and continue to proliferate throughout life. The connective tissue and mesenchymal cells are quiescent in adult mammals, but proliferate in response to injury. Fibroblasts in particular proliferate widely and constitute the connective tissue response to inflammation.

3.2.1 Control of Normal Cell Growth

Molecular events in cell growth are complex involving an array of intercellular pathways. Aberrations in such pathways may underlie the uncontrolled growth in cancer as well as abnormal cellular responses to disease processes. There are three schemes of intercellular signaling – *autocrine* (cells responding to substances they secrete themselves, *paracrine* (cells responding to substances produced in close proximity) and *endocrine* (cells responding to hormones secreted at a distant site).[8]

Certain growth factors induce cell proliferation by affecting the expression of genes controlling normal growth and are called *protooncogenes*. Aberrations in the structure of these protooncogenes convert them into *oncogenes* which lead to the uncontrolled cell growth that occurs in cancer. The processes of normal and abnormal cell division thus follow the same pathways.[9]

3.2.2 Cell Surface Receptors

Cell growth is initiated by the binding of a signaling agent or *ligand*, usually a growth factor, to a specific receptor protein. The receptor ligand complex initiates a specific cellular response. There are three major classes of cell surface receptors.

i. Receptors with intrinsic kinase activity have an extracellular domain for ligand binding, a single tramsmembrane region and a cytosolic domain which can have either tyrosine kinase activity or less commonly serine/threonine. Dimeric growth factors bind two receptors and form stable dimmers. *Dimerisation* is followed by *autophosphorylation* where one receptor molecule phosphorylates the other forming binding sites for a series of cytosolic proteins.[10] Binding of cytosolic proteins to the phosphorylated tyrosine residues on the activated receptor generate a *signal transduction* cascade which commit the cell to enter the S phase of the cell cycle.

ii. Receptors without intrinsic catalytic activity have a cytosolic domain which directly associates with and activates protein tyrosine kinases in the cytosol which in turn phosphorylates the receptor. Receptors for many cytokines fall in this category and this is termed the *cytokine receptor superfamily.*[11]

iii. G protein linked receptors contain seven transmembrane loops and are called *seven spanning receptors.* Ligand binding activates a cytosolic G protein complex that then activates an effector system generating intracellular second messengers.[12]

3.2.3 Signal Transduction Systems

Extracellular signals that are detected by the cell surface receptors are then converted into intracellular signals which generate specific cellular responses. This process is called signal transduction. Signal transduction systems are arranged as networks of sequential protein kinases. The important systems are the mitogen-activated protein (MAP) kinase, phosphoinositide-3 (PI-3) kinase, inositol lipid (IP_3), cyclic adenosine monophosphate (cAMP), the Janus kinases/signal transducers and activators of transcription (JAK/STAT) signaling system and the stress kinase system.

3.2.4 Transcription Factors

The signal information received by the cell surface receptors is transferred by the signal transducers to the nucleus where regulation of gene expression occurs. This is controlled by transcription factors which include protooncogenes and tumor suppressor genes (antioncogenes). They have a modular structure with domains for DNA binding and transcriptional regulation (regulatory domain). The DNA binding domain binds to DNA by distinct molecular mechanisms (homeodomain, zinc finger).[13] The regulatory domain either increases (activation domain) or decreases (repression domain) transcription.

3.2.5 Cell Cycle Regulation

The passage of cell through specific phases of the cell cycle is regulated by two types of molecular controls – a cascade of *protein phosphorylation* pathways involving *cyclins* and a set of *checkpoints* that monitor completion of events and sometimes delay progression to the next phase of the cycle.

Cyclins are a group of proteins that control the entry and progression of cells through the cell cycle. The cyclins (A, B and E) form complexes with specific proteins called *cyclin dependent kinases (CDKs).*[14] These complexes regulate the passage of the cells to the next stage of the cycle. The levels of the kinases peak during specific phases of the

cycle and are then degraded rapidly as the cell enters the next phase of the cell cycle[15] by the *ubiquitin-proteasome pathway.*[16] The activity of the CDK complexes is also determined by binding of *CDK inhibitors* such as p21 and p27, as well as other kinases and phosphates.[17]

Checkpoints provide a surveillance mechanism by sensing problems in DNA replication, repair and chromosome segregation.[18] When checkpoints are activated, they send signals to the cell cycle machinery that arrest the cell cycle. An example is the *p53* tumour suppressor gene that is activated in response to DNA damage and inhibits the cell cycle by increasing the expression of the CDK inhibitor, *p21.*

3.2.6 Growth Factors

Growth factors are specific polypeptides that influence the mitotic cycle. They may act on a variety of cell types or have specific target locations. They also have effects on cell locomotion, contractility and differentiation that influence wound healing. Table 3.1 lists some of the important growth factors.

3.3 Extracellular Matrix

Cell multiplication and differentiation occurs in intimate contact with the extracellular matrix (ECM). There is now evidence that the ECM critically influences these cell functions.[19] The ECM is secreted locally and forms a network of macromolecules in the spaces surrounding the cells. Three groups of macromolecules associate to form the ECM.

i. Fibrous structural proteins like collagens and elastins

ii. Adhesive glycoproteins like fibronectin and laminin

iii. A gel of proteoglycans and hyaluronan.

The ECM has many functions.[20] It sequesters water molecules to provide turgor to soft tissues and minerals to provide rigidity to skeletal tissues. It provides a reservoir for growth factors controlling cell proliferation. It also provides a base for cells to migrate, adhere and proliferate directly influencing the form and function of cells. The degradation of the ECM accompanies wound healing as well as tumour invasion.

3.3.1 Collagen

Collagens provide the extracellular framework for all multicellular organisms. They have a triple helix of three polypeptide α chains and

about 30 different chains form 14 different collagen types.[21] Types I, II and III are fibrillar and are abundant in the interstitium. Types IV, V and VI are nonfibrillar or amorphous and are present in the basement membrane and interstitium. The α chains are synthesised on the ribosome and then subjected to enzymatic modifications including hydroxylation of proline and lysine residues. Following modification, the procollagen chains align to form the triple helix. The collagens are then secreted from the cell, during which time procollagen peptidases clip the terminal propeptide chains forming *fibrils.* Specific lysine and hydroxylysine residues are oxidized by *lysine oxidase* resulting in cross-linkages between α chains of adjacent molecules stabilizing the collagen array and contributing to the tensile strength.

3.3.2 Elastic fibres

While tensile strength of the tissues is provided by collagen, the ability to recoil is provided by elastic fibres.[22] These consist of a central core made largely of *elastin*, a 70-kD protein. Similar to collagen, it is rich in glycine, proline and alanine while it contains little hydroxyproline and no hydroxylysine in contrast to collagen. The central elastin core is surrounded by a microfibrillar network mainly consisting of *fibrillin*, a 350-kD glycoprotein. Fibrillin associates either with itself or with other components of the ECM.[23] This network is the scaffolding on which elastin is laid down.

3.3.3 Adhesive Proteins.

The ECM component are linked to one another and to the cells by *adhesive glycoproteins* and *integrins.* The major adhesive glycoproteins are *fibronectin* and *laminin.* Fibronectin is directly involved in attachment, spreading and migration of cells[24] while laminin is the major glycoprotein in the basement membrane.[25]

3.3.4 Integrins

Integrins are the most important cell surface receptors that mediate cellular attachment to the ECM. They are transmembrane glycoproteins consisting of a single β chain with a series of α chains.[26] The extracellular domains of integrins bind laminin, fibronectin and many other components of the ECM by recognizing specific amino acid sequences. This causes a clustering of receptors with formation of *focal adhesions* which are links of the integrins to intracellular cytoskeleton complexes.[27] These integrin-cytoskeleton complexes function as activated receptors and activate components of the intracellular signaling systems. In this way, integrins help in organising the actin cytoskeleton as well as transduction of signals from the ECM to the cell. Integrins also play important roles in cell-cell interactions and are involeved in leukocyte adhesion and extravasation, platelet aggregation, developmental processes and wound healing.

The integrin-cytoskeleton linkage may also be the key to unraveling the *tensegrity hypothesis.*[28] Physical forces of gravity, hemodynamic stresses, and movement play a critical role in tissue development. Yet, little is known about how cells convert these mechanical signals into a chemical response. It has been suggested that the mechanical linkage between integrins and the cytoskeleton system may be the mechanism for the conversion of mechanical forces into biochemical signals[29] and this theory is under investigation.

3.3.5 Other ECM components

The other main components of the ECM are matricellular proteins, hyaluronan and proteoglycans. *Matricellular proteins* are secreted proteins that interact with matrix proteins, cell surface receptors or other molecules that in turn interact with the cell surface. *Hyaluronan* serves as a ligand for core proteins of the ECM as well as a backbone for large proteoglycan complexes.[30] Due to its capability to bind water, it forms a viscous gel that provides turgor to connective tissue as well as serves as a lubricant in the connective tissues like cartilage. It also associates with cell surface receptors that regulate cell proliferation and migration. *Proteoglycans* are a group of core proteins attached to polysaccharides called *glycosaminoglycans*.[31] Heparan sulphate, chondroitin sulphate and dermatan sulphate belong to this group and

have diverse roles in regulating connective tissue permeability and structure as well as cell growth and differentiation.

3.4 Extracellular Processes of Tissue Repair

The destruction of tissues causes damage to the cellular network as well as the stromal framework of the tissue. Even in tissues where the cells are capable of regeneration, some repair occurs by the replacement of nonregenerated cells by connective tissue. This noncellular tissue repair has three components.

i. Angiogenesis

ii. Fibrosis

iii. Remodeling

3.4.1 Angiogenesis

The formation of blood vessels is accomplished by two processes. *Vasculogenesis* involves the organisation of lakes of *angioblasts* into a primitive vascular network. *Neovascularisation* involves the formation of new vessels from capillary buds sent out from pre-existing blood vessels.[32] This involves a series of steps beginning with the proteolytic degradation of the parent vessel basement membrane and formation of

a capillary sprout. Endothelial cells then migrate towards this bud, proliferate and mature with the inhibition of further growth and the formation of capillary tubes. Peri-endothelial cells like smooth muscle cells are then recruited to provide support to the endothelial tubes.

These steps are controlled by interactions between growth factors, vascular cells and the ECM. The most important growth factor in adult tissues undergoing physiological angiogenesis is the family of *Vascular Endothelial Growth Factors (VEGF).* An embryological model[33] has been developed that elucidates the important role of VEGF in promoting angiogenesis, increasing vascular permeability, stimulating endothelial cell migration and proliferation and regulating the expression of other growth factors.

The *integrins* and *matricellular proteins* also are involved in the control of the motility and migration of the endothelial cells. Certain proteases like *plasminogen activators* and *matrix metalloproteinases* play an important role in the remodeling that occurs during endothelial invasion as well as in cleaving extracellular proteins to form fragments like *endostatin* that regulate angiogenesis.

Table 1. Growth factors – Their Source and Primary Activity

Factor	Principal Source	Primary Activity	Comments
PDGF	platelets, endothelial cells, placenta	promotes proliferation of connective tissue, glial and smooth muscle cells	two different protein chains form 3 distinct dimer forms; AA, AB and BB
EGF	submaxillary gland, Brunners gland	promotes proliferation of mesenchymal, glial and epithelial cells	
TGF-α	common in transformed cells	may be important for normal wound healing	related to EGF
FGF	wide range of cells; protein is associated with the ECM	promotes proliferation of many cells; inhibits some stem cells; induces mesoderm to form in early embryos	at least 19 family members, 4 distinct receptors
NGF		promotes neurite outgrowth and neural cell survival	several related proteins first identified as proto-oncogenes; trkA (trackA), trkB, trkC
Erythropoietin	Kidney	promotes proliferation and differentiation of erythrocytes	
TGF-β	activated TH_1 cells (T-helper) and natural killer (NK) cells	anti-inflammatory (suppresses cytokine production and class II MHC expression), promotes wound healing, inhibits macrophage and lymphocyte proliferation	at least 100 different family members
IGF-I	primarily liver	promotes proliferation of many cell types	related to IGF-II and proinsulin, also called Somatomedin C
IGF-II	variety of cells	promotes proliferation of many cell types primarily of fetal origin	related to IGF-I and proinsulin

3.4.2 Fibrosis

Within 24 hours following tissue injury, fibroblasts and vascular endothelial cells begin proliferating to form a specialised tissue called *granulation tissue* that is the hallmark of healing. It is within this tissue that fibrosis begins. There are two processes involved in fibrosis. The initial step is the migration of fibroblasts to the site of the injury followed by their proliferation. The second step is the deposition of ECM by the fibroblasts.

The migration and proliferation of fibroblasts are triggered by a number of growth factors given in Table 1. These growth factors are derived from platelets, activated endothelium and a variety of inflammatory cells like macrophages, mast cells, eosinophils and lymphocytes. The most important growth factor in fibrosis is TGF-β. TGF-β is produced by most cells present in granulation tissue and has many different actions. It causes fibroblast migration and proliferation, increased synthesis of collagen and fibronectin, decreased degradation of ECM by metalloproteinases, migration of monocytes and stimulating angiogenesis.

The migration and proliferation of fibroblasts gradually decreases and they begin to synthesise and deposit ECM components, especially fibrillar collagens, on the granulation tissue. This process continues for

many weeks depending on the size of the wound. The growth factors regulating fibroblast proliferation now influence collagen formation. The net accumulation of collagen depends on collagen synthesis as well as degradation, both of which occur simultaneously. Vascular regression takes place and the granulation tissue is gradually converted into an avascular scar.

3.4.3 Remodeling

The transformation of granulation tissue to scar tissue involves a number of changes in the ECM that are mainly brought about by the synthesis and degradation of collagen. These two processes that occur simultaneously bring about remodeling of the connective tissue and stable scar formation. The degradation of collagen is brought about by a group of endopeptidases called *matrix metalloproteinases (MMPs).* Activated MMPs are inhibited by a family of *tissue inhibitors of metalloproteinase (TIMP)* which are produced by most mesenchymal cells. The equilibrium between MMPs and TIMPs is a key factor in tissue remodelling.

3.5 Matrix Metalloproteinases (Matrixins)

Matrix metalloproteinases (MMPs), otherwise called *matrixins,* are a group of endopeptidases that play a vital role in the healing process. They are collagenases and act by breaking down collagen. This function of breakdown of extracellular matrix (ECM) is essential for embryonic development, morphogenesis, reproduction, and tissue resorption and remodeling[34]. Coordinate synthesis and degradation of ECM components is also necessary for many important pathophysiological processes involved in inflammation, such as leucocyte invasion,[35] epithelial migration,[36,37] neovascularisation,[38] and wound healing.[39] Disturbance of the balance between synthesis and degradation of ECM components may result either in progressive organ destruction, as seen for example in ulcer formation,[40] or excessive deposition of collagens resulting in fibrosis.[41] The expression of most matrixins is transcriptionally regulated by growth factors, hormones, cytokines, and cellular transformation[42]. The proteolytic activities of MMPs are precisely controlled during activation from their precursors and inhibition by endogenous inhibitors, macroglobulins, and tissue inhibitors of metalloproteinases (TIMPs). The first MMP activity discovered was a collagenase in the tail of a tadpole undergoing metamorphosis. To date, 24 different vertebrate MMPs have been identified, of which 23 are found in humans.[43]

Table 2

Vertebrate members of the matrixin family

See Appendix 3 for domain composition.

Protein	MMP	Domain composition
Collagenase 1	MMP-1	B
Gelatinase A	MMP-2	C
Stromelysin 1	MMP-3	B
Matrilysin	MMP-7	A
Collagenase 2	MMP-8	B
Gelatinase B	MMP-9	D
Stromelysin 2	MMP-10	B
Stromelysin 3	MMP-11	E
Macrophage elastase	MMP-12	B
Collagenase 3	MMP-13	B
MT1-MMP	MMP-14	F
MT2-MMP	MMP-15	F
MT3-MMP	MMP-16	F
MT4-MMP	MMP-17	F
Collagenase 4 (*Xenopus*)	MMP-18	B
(No trivial name)	MMP-19	B
Enamelysin	MMP-20	B
XMMP (*Xenopus*)	MMP-21	G
CMMP (chicken)	MMP-22	B
(No trivial name)	MMP-23	H

Courtesy. Nagase H, Woessner JF Jr. Matrix Metalloproteinases. J Biol Chem. 1999; Jul 30; 274(31):21491-4.

The sequence homology with collagenase 1 (MMP-1), the cysteine switch motif in the propeptide that maintains MMPs in their zymogen form (proMMP), and a zinc-binding motif in the catalytic domain are the signatures used to assign proteinases to this family. MMPs generally consist of a prodomain, a catalytic domain, a hinge region, and a hemopexin domain (see Appendix 3). On the basis of substrate specificity, sequence similarity, and domain organization, vertebrate MMPs can be divided into six groups – *collagenases, gelatinases, stromelysisn, matrilysins, membrane bound MMPs* and *other MMPs* (see Table 2). The complex three dimensional structure of a few MMPs have been described using X-ray crystallography and nuclear magnetic resonance (NMR) and is shown in Appendix 2.

The activaton of proMMPs to MMPs takes place in a stepwise fashion either by proteinases *in vivo* or by a number of chemical agents *in vitro.* Most activation occurs extracellularly, but recently, there have been reports of intracellular activation as well as cell surface activation. Following activation, the MMPs fulfil a number of roles in cellular physiology. A major function of MMPs is thought to be the removal of ECM in tissue resorption. However, the ECM is not simply an extracellular scaffold; it also acts as a reservoir of biologically active molecules, such as growth factors.[44] Some ECM components can express cryptic biological functions on proteolysis. Hence, degradation

of ECM components by MMPs can alter cellular behavior and phenotypes (Table 2). Add to this the ever expanding number of non-ECM proteins that are MMP substrates and exert biological activities[45], and the complexity of the role of MMPs in health and disease is evident.

Recently, MMPs have been implicated as one of the main factors contributing to mucosal ulceration in inflammatory bowel disease (IBD).[46,47,48,49,50,51] Although these diseases are undoubtedly caused by excessive immune reactivity in the mucosa, the end stage effector molecules for mucosal degradation appear to be the MMPs secreted by cytokine activated stromal cells.[52] There have also been reports on the role of MMPs in colonic anastomotic healing. Excessive matrix metalloproteinase activities have been implicated in the pathogenesis of intestinal anastomotic dehiscence[53] and synthetic metalloproteinase inhibitors like *marimastat* are being studied in a therapeutic role[54]

3.6 Tissue Inhibitors of Metalloprotinases (TIMPs)

TIMPs (21-30 kDa) are the major endogenous regulators of MMP activities in the tissue, and four homologous TIMPs (TIMPs 1 to 4) have been identified to date.[55] The crystal structure of the complex formed

between TIMP-1 and the catalytic domain of MMP-3 was first determined by Gomis-Rüth *et al*.[56]

TIMPs form classic noncovalent bimolecular complexes with the active forms of MMPs and under certain circumstances with their latent forms as well. TIMPs inhibit the activity of the fully competent MMP and also appear to block or retard MMP precursor activation.[57,58] The role played by TIMPs in regulating matrix degradation may therefore be exerted not only by classic proteinase elimination but also by a perhaps even more potent effect, namely, the interceptive blockage of MMP activation. TIMPs appear to be distributed widely in tissues and fluids[59,60] and to be expressed by many different normal and transformed cell types.

TIMPs have been found to play a role in many cell physioplogical functions especially tumorigenesis, carcinogenesis, metastasis and angiogenesis. TIMPs have been found to have mitogenic effects on many cells (especially tumour cells) by inhibiting apoptosis though they inhibit tumour cell growth when overexpressed. These effects are in addition to the biological functions influenced by their role in inhibiting MMPs and are in fact independent of MMPs.[61,62] TIMPs have also been found to be involved in the pathogenesis of ulcerative colitis along with MMPs with Louis et al finding increased expression of TIMP-1 at ulcerated sites of mucosa, but no increased expression in either

controls or patients with UC.[63] The significant correlation of TIMP-1 levels with clinical and endoscopical signs of UC activity have led to the suggestion that it may be used as a biomarker of the disease.[64]

3.6 Processes of Wound Healing with Specific Reference to Colonic Healing

Wound healing is a series of events that take place following tissue injury in an orderly manner. This process has the same basic steps in all human tissue, though the healing in different organs may differ in certain processes as individual organs contain specialised cells that provide organ specificity to the healing process. The basic steps involved in wound healing are

i. Initiation of inflammation by injury

ii. Parenchymal cell regeneration

iii. Migration and proliferation of parenchymal and connective tissue cell to the site of injury

iv. Synthesis of proteins and laying down of ECM

v. Remodelling

vi. Organisation of collagen providing wound strength.

3.6.1 Anatomical and Physiological Considerations

The wall of the intestine histologically consists of four layers – mucosa, submucosa, muscularis propria and serosa. These are seen in the whole gut tube from the esophagus to the rectum reflecting the general structural organisation of the entire GI tract.

The innermost layer of the colon wall is the *mucosa*, which is essentially the same in the whole colon. This layer consists of a layer of nonkeratinising stratified squamous *epithelium* overlying the *lamina propria mucosa.* The laminal propria contains blood vessels, lymphatics and nerve endings as well as a number of mesenchymal cells and inflammatory cells such as macrophages, lymphocytes and mast cells. Surrounding the lamina propria is the muscularis mucosae, a thin, vertical muscle layer. In contrast to the small intestine, the colonic mucosa has no villi and is flat. The mucosa is punctuated with numerous straight tubular crypts that extend down to the muscularis mucosa. The surface epithelium is composed of columnar absorptive cells which have shorter, less abundant microvilli than the small intestine and goblet cells. The crypts contain abundant goblet cells, endocrine cells and indifferentiated crypt cells. Paneth cells are occasionally present in the base of the crypts in the caecum and ascending colon.

The mucosal lining of the gastrointestinal tract is composed of a rapidly proliferating and continually renewing sheet of epithelial cells that serve a variety of specialized physiologic functions including selective absorption of nutrients, surface hydration, immune surveillance, and the formation of a permeability barrier between the hostile luminal environment and the body interior. In the intestine, the epithelial cells that carry out these tasks are anatomically compartmentalized and derive from a population of rapidly proliferating stem cells located several cell positions above the base of the crypts. Over the course of a 4- to 6-day lifespan, undifferentiated crypt cells migrate out of the proliferative crypt zone along the so-called crypt-villus axis, where they acquire phenotypic characteristics of mature absorptive enterocytes before undergoing apoptotic cell death.[65]

The *submucosa* is the most important layer in the GI tract with regard to anastomotic healing, since it is the support for the sutures.[66] It is composed of loose connective tissue containing the majority of matrix proteins. The composition of the matrix, however, is different from that of skin. It contains large amounts of collagen type I, less of type III collagen, and also type V collagen. Smooth muscle cells are the predominant matrix producers. The submucosa is richly vascularized, containing an arterial and venous plexus as well as a network of lymphatics.

The *muscularis propria* consist of two thick layers of smooth muscle cells, an inner circular layer and outer longitudinal layer. The *serosa* or the visceral peritoneum contains mesothelial cells on a thin layer of mesenchymal cells and matrix. It is rich in vasculature and lymphatics.

The same four layers exist throughout the whole GI tract. The esophagus and rectum lack serosal coverage: They are the site of increased risk of anastomotic failure as well as increased stricture formation.

The colon reabsorbs water, electrolytes (sodium, chloride), and short chain fatty acids (butyrate). It further secretes mucus to soften the feces. In contrast to the small bowel, the colon contains large amounts of aerobic and anaerobic bacteria. One gram of feces contains approximately 1011 bacteria, which represents 3% of the stool mass.[67]

3.6.2 Phases of Healing.

The three phases of GI healing are inflammation, proliferation, and tissue remodeling. As with reepithelialization of the skin, if the mucosa is the only injured layer, it can be reformed by migration and proliferation. Full-thickness injury provokes a fibroblastic response resulting in scar formation.

The inflammatory phase begins immediately after the injury and is characterized by cell infiltration and edema formation. Edema is mainly confined to the submucosa.[68] It subsides completely only by the end of the second week. Granulocytes are the predominant cell type in the first 24 hrs, and macrophages follow 48 hrs after clean surgical injury. Fibroblasts and smooth muscle cells are recruited and produce matrix proteins for laying down of ECM in the submucosa, subserosa and serosa.[69,70] Smooth muscle cells contribute more to absolute collagen formation than fibroblasts.

Mucosal resurfacing is rapid in the GI tract and mucosal continuity is fully reestablished within 1-2 weeks, over a base of granulomatous tissue consisting of proliferating smooth muscle cells and fibroblasts.[70] However, if the injury is severe or is accompanied by tissue destruction or bacterial spillage, the process may take longer. Angiogenesis starts as early as 2–3 days after injury,[71] accompanied by reconstitution of the submucosal collagenous network which is important for epithelial regeneration. This is followed by remodeling and thinning of the anastomosis. The muscularis mucosae and muscularis propria stay disorganized, however, allowing the repair process to be recognized even 1 yr after operation.[69]

3.6.3 Anastomotic Strength.

The continuity of the colon is reestablished by deposition of new collagen based matrix that bridges the anastomosis. Collagen types I, III, and V are the major isotypes during colon repair, type I being the predominant one. In general, collagen accumulation and deposition represent the balance between new synthesis and degradation.

3.6.4 Bursting Pressure.

Immediately after its creation, and for 2–3 days thereafter, the anastomosis is held together by surgically placed sutures and sealed by a fibrin clot. During this time period, there is little or no new matrix synthesis, but there is marked and sustained lysis of the structural collagen matrix. After 3 days there is a noticeable increase in strength of the anastomosis.[68] After day 7, experimental anastomoses acquire their full mechanical strength and rupture outside the suture site (Fig. 2) (5). This is owing to the rigidity of the newly established connection compared with the adjacent bowel and to an increase in bursting strength of the colon remote from the anastomosis.[69]

3.6.5 Collagen Synthesis and Collagenase Breakdown.

Fibronectin, a high molecular weight glycoprotein, usually is expressed at low concentrations in the healthy submucosa. Several hours after injury, its expression is induced in the submucosa, serosa, and muscle layers; the expression is strongest around days 3–5. Fibronectin serves as a scaffold for infiltrating cells and as provisional matrix. The expression declines during the proliferative phase.[70] Basal expression of collagen I and II is found in the lamina propria and submucosa of the uninjured colon. After injury, collagen gene expression, as measured by Northern blotting, is strongly activated with a peak around day 3 for collagen I and around day 2 for collagen III.[71] Immunohistochemical staining, however, shows a strong decline in staining for both collagens at days 2 and 3 with a gradual increase until day 7.[74] This loss of collagen is most marked in the proximal and distal 2.5 cm from the anastomosis[73] and signifies increased breakdown of collagen by collagenases like MMPs. In the basal lamina, type V collagen as well as laminin shows a similar pattern of staining. Small-bowel anastomoses have a faster and more pronounced collagen synthesis compared with colon anastomoses.[72] Furthermore, collagen breakdown is almost negligible in small-bowel anastomoses compared with the colon.[71,73,76] This may account for the lower failure rate of small-bowel anastomoses.[73]

The synthesis and deposition of collagen as well as degradation of preformed collagen occur simultaneously in the healing process anddetermine wound strength and the balance between these two factors determines the strength and integrity of the wound in the early period following injury.[74] The initial breakdown of collagens at the anastomosis is partially due to an increased collagenase activity.[72] Specific collagenases break up the triple helix and render it more susceptible to breakdown by other enzymes. The activity of the collagenases is regulated by their counterparts, the tissue inhibitors of metalloproteinases. Collagenases are induced within a few hours after injury and are strictly located at the anastomosis,[114] followed shortly by tissue inhibitors of metalloproteinases which regulate collagenase activity.[75] The main sources of collagenases are the granulocytes and fibroblasts and also the intraluminal. Overproduction of collagenases during infections or abscess formation can lead to higher dehiscence rate.[76]

The data on collagenase inhibitors and colon healing are controversial as both positive and negative effects have been shown. It has to be kept in mind, however, that synthetic inhibitors of metalloproteinases have other collateral and distinct functions such as inhibition of tumour necrosis factor-α formation[77] and inhibition of angiogenesis.[78] A recent human study demonstrated that resected colon tissue specimens from

patients who subsequently developed an anastomotic leak had decreased collagen I and III concentrations and decreased overall collagen content compared with normal healing controls.[79] There was also an increased detection rate of matrix metalloproteinase-13 in the anastomotic failure group. Since the differences in collagen expression were already present at the time of the operation, it suggests that there may be genotypic or phenotypic alterations in bowel wall collagen composition that could explain why even under the best conditions some anastomoses fail to heal with no obvious clinical risk factor being present.

3.6.6 Growth Factor Expression.

The expression of growth factors with regard to healing of colonic anastomoses has not yet been well investigated. Some data are available on mucosal repair during colitis[80] and anastomotic healing.[81] A few of the growth factors possibly involved in colonic healing are mentioned below.

Epidermal growth factor is a potent stimulator of cell proliferation in the GI tract. It promotes intestinal repair and modulates polyamine synthesis. The receptor for epidermal growth factor is widely expressed in the intact GI tract, and increased expression is detected in colonic epithelium and fibroblasts around day 7 after surgery. However, the

complementary DNA for epidermal growth factor is not up-regulated at the anastomosis.[70] More research is required to identify if epidermal growth factor plays a role in anastomotic healing.

Transforming growth factor (TGF)-β is mainly produced in the epithelial layer in the intestine. It inhibits the proliferation of fibroblasts and epithelial cells, modulates lymphocyte function, and induces and stimulates fibroblast matrix synthesis. TGF-β knockout mice have diffuse intestinal inflammation. TGF-β expression in healing colon anastomoses correlates with increased collagen I expression[85] and Single TGF-β application accelerates gastric incisional healing in a rabbit model[82] underscoring the significance of TGF-β in bowel healing.

Insulin-like growth factor (IGF) enhances epithelial repair and stimulates cell proliferation. Intraperitoneal or systemic administration of IGF-I increases bursting pressure of anastomoses,[83] but this effect may possibly be due to enhanced collagen deposition.[84] However, growth hormone, which mediates IGF release, improves colon healing by increasing collagen deposition in a dose-dependent manner.[85]

Keratinocyte growth factor (KGF) is synthesized by stromal fibroblasts but acts in a paracrine manner on epithelial proliferation. KGF receptor and messenger RNA are expressed throughout the entire intestine. During colon anastomotic healing, KGF increases bursting pressure

during the first postoperative week. KGF reduces local inflammation and increases epithelial proliferation but does not increase collagen synthesis at the anastomosis.[86] A combination of KGF and IGF has synergistic effects on the healing of experimental colon anastomoses.[87]

3.7 Butyrate and its Role in Colonic Healing

Butyrate is a four carbon molecule formed from bacterial fermentation of complex carbohydrates in the colon. It has a variety of effects with reference to colonocyte nutrition, colonic epithelial physiology and disease.

Butyrate is the most important Short Chain Fatty Acid (SCFA) and is preferentially metabolized by colonic mucosa when compared to propionate and acetate. The dependence of the colon related to the oxidation of SCFA increases towards the rectum, and 70% of the oxygen consumed by the colonic epithelial cells is used in the oxidation of SCFA.[87]

The intraluminal environment of the colon differs from the rest of the intestinal tract due to the presence of a variety of bacteria that colonise the colon. These organisms are mainly anaerobic and digest the undigested food products in the effluent delivered to the colon,

especially the complex sugars contained in dietary fibre. This fermentation of sugars is an important source of colonic gases such as hydrogen, methane and carbon dioxide. Digestion of bile salts in malabsorbed long-chain fatty acids leads to the formation of potent secretagogues which causes diarrhoea.

The fermentation of complex sugars by the colonic bacteria leads to the formation of short-chain fatty acids (SCFAs), namely, butyrate, propionate and acetate. These SCFAs, especially butyrate are important sources of colonic nutrition with butyrate providing upto 80% of colonocyte energy. They are actively and passively transported into the cell where they provide energy through the β-oxidation pathway. Fermentation of dietary fibre by colonic bacteria can provide upto 500 calories/day of the overall nutritional needs.[88]

The physiological role of butyrate and the other SCFAs in the colon has been the reason that much interest has been generated over their possible role in colonic healing. Recently, there have been many studies looking at the role of butyrate in the pathophysiology and treatment of colonic disease and injury. There have been encouraging results and the role of butyrate in colonic healing has now been firmly established.

It has been found that a covering stoma weakened colonic anastomoses in rats leading to higher leak rates as identified by radiological studies.[89] This could be attributed to the absence of short chain fatty acids (SCFAs) in the colon due to diversion, though more confirmatory research is required. It has also been found that butyrate enemas strengthened the anastomoses in rats exposed to radiotherapy.[90]

Earlier researchers have proved that a continuous infusion of a mixture of SCFAs significantly strengthened a left colonic anastomosis in rats.[91] This study by Rolandelli et al used a mixture of acetate, propionate and butyrate as a continuous infusion through the proximal colon to bathe a distal colonic anastomosis. They found a significant difference in the bursting pressures as well as the bursting wall tension of the anastomoses when compared to controls that were given a solution of electrolytes.

Continuous infusion of solution is not a feasible clinical option and it was necessary to identify usable routes to provide SCFA to the healing anastomosis. A further study by Rolandelli et al found that intravenous infusion of butyrate also improved the mechanical strength of colonic anastomosis with significant increase in bursting pressure and bursting wall tension over a group not given butyrate.[92]

There has been much research into the role of butyrate in the pathogenesis and treatment of ulcerative colitis (UC). Damage to colonic epithelial cells with impaired cell metabolism and barrier function is a hallmark of active UC.[93] In drug induced UC, butyrate has been found to have a definite cytoprotective effect.[94,95] Butyrate has been used experimentally with favourable results in the treatment of ulcerative colitis.[96,97,98,99] The therapeutic efficacy of butyrate could be explained on the basis of the fact that butyrate serves as the major source of energy for colonocytes, increases mucosal proliferation, promotes cell differentiation, and improves barrier function.[91] It has been assumed that the protective effect of butyrate in these diseases results predominantly from influencing energy availability in the colonocyte, because it is the primary source of energy in these cells .[100] However, the cellular signaling events initiated by butyrate to carry out its protective effect have not been completely elucidated.

Diversion colitis is a condition that frequently develops in segments of the colorectum after surgical diversion of the fecal stream. It persists indefinitely unless the excluded segment is reanastomosed and probably represents an inflammatory state resulting from a nutritional deficiency in the lumen of the colonic epithelium. Harig et al have demonstrated amelioration of symptoms and healing of the colonic epithelium with instillation of a solution of SCFAs over a period of four

to six weeks.[101] Radiation proctocolitis is a complication of radiation therapy to the pelvis for malignant disease. The acute haemorrhagic form is the most severe and can be fatal. This disease has been resistant to the many forms of therapy tried. There is now, however, evidence showing the effectiveness of intraluminal butyrate infusion in the treatment of this condition.[102] This is another piece of experimental evidence to suggest the role of butyrate in colonic healing.

It has been postulated that impaired healing in the colon may result from an increased breakdown of collagen by increased activity of collagenases.[103] These collagenases are produced in the colon by the intraluminal bacteria and the endotoxins secreted by these bacteria activate collagenases. As butyrate is the preferred form of nutrition for the colonocyte,[104] it has been the subject of much recent study for a possible role in facilitating early colonic healing.

4.0 Materials and Methods

4.1 Animal Experiments

Forty male albino rats of the Wistar strain weighing between 200 – 250 grams were used for the experiment. The animals were fed a fibre-free diet to minimise the production of SCFAs from the fibre in the colon. This was begun 2 days before the initial operation and continued till the end of the experiment. The diet used was a specially prepared purified fibre free diet based on the American Institute of Nutrition (AIN)-93M diet.[105] The diet was prepared initially in a powder form and reconstituted every day with water to make a semi-solid diet.

After 48 hours, the rats were anaesthetised using ether chamber and intra-peritoneal ketamine (50mg/kg) and a midline laparotomy was done. The colon was first washed out with normal saline and then transected in two places preserving the marginal vessels (Fig 6.1). The ascending colon was transected about 3 cms from the ileo-caecal junction and the descending colon about 3 cms from the peritoneal reflection. Specimens were taken from both areas, frozen immediately in liquid nitrogen and stored at -70˚C. An end-to-end anastomosis was carried out with a single layer of interrupted 6. 0 proline sutures (Fig 6.2, 6.3). A diversion caecostomy was carried out and the abdomen closed in layers.

The animals were then injected 20 ml of saline subcutaneously to prevent post-operative dehydration. The rats were continued on the fibre-free diet for the remainder of the experiment.

The rats were randomly assigned to two groups – butyrate and control. The rats in the butyrate group were given 10 ml of 140 mmol solution containing 80 mmol of butyrate and 60 mmol of sodium chloride as an enema daily beginning from the third post-operative day. The rats in the control group were given 10 ml of 140 mmol sodium chloride enemas. The enemas were instilled in the rectum using a non-traumatic plastic cannula.

The rats were anaesthetised again on the seventh post operative day and the bursting pressure was measured *in vivo* without disturbing the adhesions around the anastomosis using an electronic transducer (Hewlett Packard, 78353B) (Fig 6.4, 6.5). The colon was infused with saline at a constant rate and the pressure curve was observed on the monitor. At the point when the colon burst, there was a sudden drop in pressure as evidenced by the pressure curve and this pressure was taken as the bursting pressure. It was noted in each case whether the colon burst at the anastomosis or away from it.

The anastomotic site was then transected longitudinally including 1 cm from the anastomosis on each side. The anastomotic circumference

was then measured. Samples were taken from the anastomotic sites as well as a virgin area of colon and frozen and stored as before. The rats were then sacrificed by transecting the inferior venacava. The rats were stored in cages housing 4 animals pre-operatively and in individual cages post-operatively. The methods used were examined by the institutional animal ethics committee and the use of the animals was sanctioned.

4.2 Calculation of Bursting Wall Tension

There are two described methods of testing experimental anastomoses. The first is the identification of breakdown or leakage by radiological methods that has been recently adapted to animal models.[106] The second method is the testing of the mechanical strength of the anastomosis. This can be done in two ways – by applying a longitudinal stretch force or by increasing intraluminal pressure. It has been found that increasing the intraluminal pressure provides a better reflection of the physiological strain that longitudinal stretching.[107,108] It has also been shown that the bowel wall tension best characterises the forces responsible for loss of anastomotic continuity.[109]

The strength of the anastomosis in this study was measured by the Bursting Wall Tension (BWT) in dyne10^{-3}/cm based on Laplace's law. It was calculated using the Bursting Pressure (BP) and the anastomotic circumference.

$$BWT = \frac{BP \times 1.36 \times \text{anastomotic circumference}}{2\pi}$$

4.3 Statistical Analysis

The results were tabulated on a spreadsheet and statistical analysis was done using SPSS software.

4.4 Estimation of Matrix Metalloproteinases (MMPs)

The estimation of MMPs was carried out by zymography as described by Rosario et al.[110] There were 5 specimens for each animal in the study – two specimens from the distal and proximal colon taken at the first laparotomy and 3 specimens taken at the second laparotomy from the distal and proximal anastomosis and from a virgin area of colon between the two anastomoses. The specimens were frozen using liquid nitrogen and stored at -70˚C at operation.

4.4.1 Homogenisation

At the time of MMP estimation, they were washed with cold phosphate-buffered saline (PBS) to remove any blood clots of attached fat. They were then hand-homogenised on ice with PBS and a serine and cysteine protease inhibitor cocktail (4,2 Amino-ethyl benzene sulphonyl hydrochloride with leupeptin, aprotinin and E64). This cocktail inhibits the serine and cysteine proteases which cleave the MMPs. They are inactive when the samples are stored at -70˚C and begin to act once the temperature rises after removal from storage. The fat and other proteins were removed and the homogenate was centrifuged for half an hour at 14000 G (13,400 RPM) (Fig. 6.6).

4.4.2 Protein estimation

The amount of protein present in the homogenate was then estimated using Lowry's protein assay and bovine serum albumin as a standard. The amount of protein in the homogenate would differ based on the initial weight of the specimen. Hence, a protein assay was necessary before running the gels in order to provide a constant weight of protein from all the specimens. Lowrys reagent (Copper Sulphate, Sodium potassium tartrate, Sodium bicarbonate and Sodium hydroxide) was added to 5 µl of the specimen and incubated for 10 minutes. Folin's (phosphor-18-tungstic acid) phenol reagent was then added and

incubated for half an hour. The protein content was then estimated by spectrophotometry.

4.4.3 Preparation of the Gel

The gel used for zymography is based on the polymerisation of acrilamide and bis-acrilamide. The polymerisation converts the liquid constituents into the gel. Following standardisation, a 1% gelatine based gel was used. 10 µg of gelatine was dissolved in 5 ml of water and added to 2.5 ml each of pre-prepared solutions A and C. Solution A contained stable acrilamide and bis-acrilamide and Solution C contained stable Temed, Sodium dodecyl suphate (SDS) and TrisH. Then 10 mg of Ammonium persulphate was added to the solution and the liquid loaded into a Biorad electrophoresis assembly between plates. A solvent is used to remove any bubbles that may have formed. The gel is formed by the polymerisation of acrilamide and bis-acrilamide. The Temed is first made unstable by the ammonium persulphate. This ionises the acrilamide and bis-acrilamide and they polymerise when in the ionised state. The process takes around 10 minutes following which the gel sets.

The loading gel was then added. This has a smaller concentration of acrilamide and bis-acrilamide with glycinate and chloride. The purpose is to suspend the sample proteins between the glycinate and chloride

so that they move down the gel in a constriction band. A template was used to make the wells and after setting, the assembly was stored at 4˚C till use.

4.4.4 Loading the Specimens

The specimen proteins were than added to a running buffer to make up 10µl and 5µl of loading buffer were added to make 15µl. The loading buffer contained Bromophenol blue, SDS, TRIS and glycerol. The running buffer contained SDS, TRIS and glycine. The mixture of sample, loading buffer and running buffer were then loaded in the wells along with a commercially available protein mix containing proteins of different molecular weights as a control (Fig. 6.7, 6.8). The samples were loaded at 4˚C and were then electrophoresed at 120V for one and a half hours at 4˚C (Fig 6.9).

4.4.5 Incubation

After electrophoresis, the gels were washed and Triton X, a commercially prepared detergent was added and incubated for one and a half hours. Triton X has two functions. It excludes the SDS from the gel and denatures it and also renatures MMPs. SDS in the gel denature MMPs causing them to fold. This deactivates them preventing any action of the MMPs on the gel during electrophoresis. Running the electrophoresis at 4˚C also prevents action of the MMPs as the ideal

temperature for the action of MMPs is 37˚C. Once the electrophoresis is complete, the proteins would have moved to their respective zones based on the molecular weight. Triton X then removes the SDS which allows renaturation of the MMPs. The gels were then incubated for 18 hours at 37˚C and a pH of 7.5 with an incubation buffer containing TRIS, Calcium chloride and BRIG (a commercial detergent). Calcium chloride also acts as an activator of MMPs. After incubation, the gels are stained with a stain containing methanol, acetic acid, Coomassie brilliant blue and water (Fig. 6.10). They were then de-stained and the gels were read. The MMPs digest the gelatine in the gel during the incubation period and this is seen as a band of clearing in the blue stained gel.

5.0 Results

5.1 Experimental results

Of the 40 rats used, 9 died during the course of the

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Of the 40 rats used, 9 died during the course of the experiment. Six rats died during anaesthesia or in the immediate post-operative period. Of these, 4 animals died in the ether chamber possibly due to a reaction to ether or a vasovagal attack. One animal did not wake up after the operation and another animal died of acute cardiac tamponade as the post operative dose of saline was injected into the thorax instead of subcutaneously. Three animals died during the post operative period. One died on the first post-operative day possibly due to aspiration and two died on the fifth post-operative day due to intestinal obstruction. There were no demonstrable anastomotic leaks during the study.

5.2 Animals Excluded from the Study

Of the 31 animals that survived till the seventh post-operative day, 8 animals could not be used for the study. Of these, 2 animals missed the enemas on certain days and 2 animals were not fed the fibre-free diet on certain days. While standardising the procedure for testing the bursting pressure, there were gross errors in 4 animals leading to their exclusion from the study.

5.3 Strength of the anastomoses

The mean Bursting Pressure (BP) for the animals given saline was 150.5 mm of Hg for the ascending colon and 168.54 mm of Hg for the descending colon. The mean BP for the animals given butyrate was 168.54 mm of Hg for the ascending colon and 188.62 mm of Hg for the descending colon (Chart 7.1). The difference in the means between the butyrate and saline arms was not statistically significant with p values of 0.3 for the ascending colon and 0.06 for the descending colon.

The mean anastomotic circumference was 1.37 cm for the ascending colon and 1.47 cm for the descending colon in the rats given saline and 1.64 cm and 1.68 cm for the ascending and descending colon respectively for the rats given butyrate (Chart 7.2). The difference in

the means between the butyrate and saline arms was not statistically significant with p values of 0.03 and 0.27 for the ascending and descending colon respectively.

The bursting wall tension (BWT) for each anastomosis was calculated from the bursting pressure and the anastomotic circumference using the formula given above based on Laplace's Law. It was found that the mean BWT of the proximal colonic anastomosis was 48.9 dyne10^{-3}/cm for rats given saline and 64.71 dyne10^{-3}/cm for rats given butyrate (Chart 7.3). The distal colonic anastomotic BWT was 51.44 dyne10^{-3}/cm for rats given saline and 72.38 dyne10^{-3}/cm for rats given butyrate (Chart 7.4). There was a significant difference in the BWTs of animals in the butyrate and control group as calculated by the Student's t-test with p values of 0.04 for the ascending colon and 0.01 for the descending colon.

It was also noted that the colon burst away from the anastomosis in 36 out of 46 anastomoses,

5.4 Matrix Metalloproteinase Zymography

The zymogram of the MMPs showed increased activity of MMP 2 in all the post operative samples as evidenced by increased clearance of the

blue-stained gelatine (Fig 6.11, 6.12). The increased activity was more pronounced in the animals given butyrate at the sites of the anastomoses. There was a smaller increase in the animals given saline as well as at the virgin area of colon. However, there was an increased activity of MMP 9 in the post operative specimens of animals given saline when compared to butyrate.

6.0 Figures

Figure 6.1.

Figure 6.1. The proximal colon before transaction.

Figure 6.2.

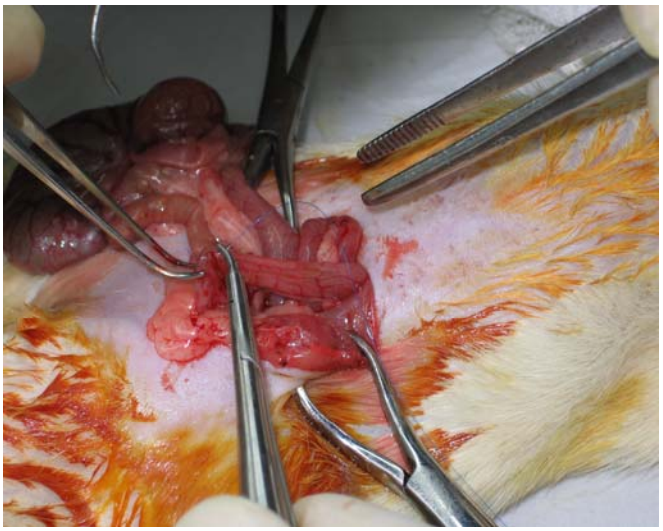


Figure 6.2. Single layered anastomosis sutured with interrupted 6/0 proline sutures.

Figure 6.3.

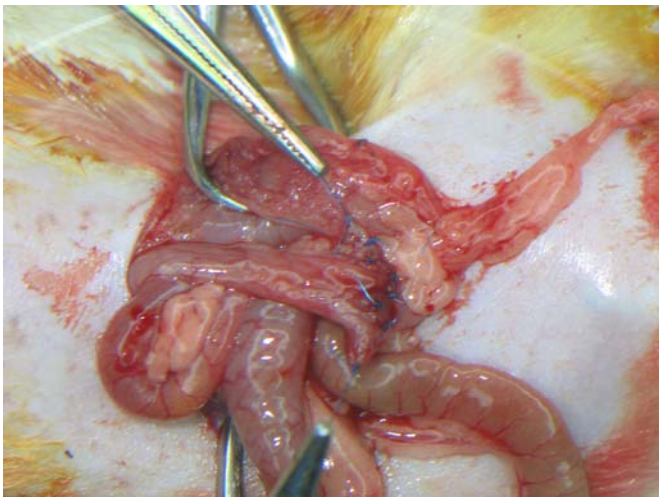


Figure 6.3. The completed anastomoses.

Figure 6.4.

Figure 6.4. Bursting pressure measurement

Figure 6.5.

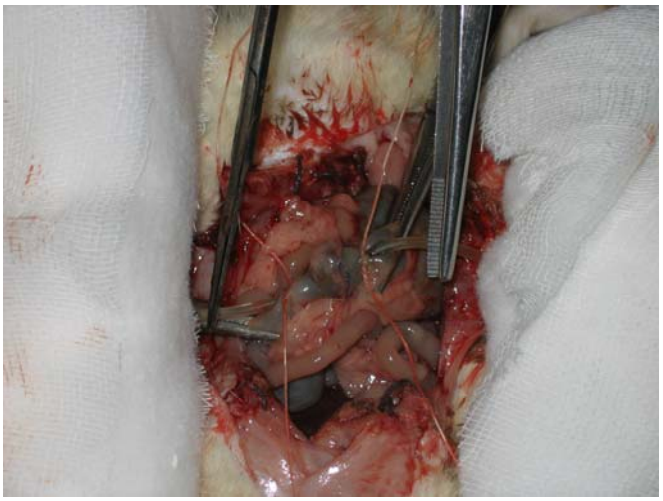


Figure 6.5. Bursting pressure measurement. Infusion catheter and pressure catheter are seen on either sides of the anastomosis. Small bowel and omental adhesions are noted around the bowel.

Figure 6.6.

Figure 6.6. Centrifuging

Figure 6.7.

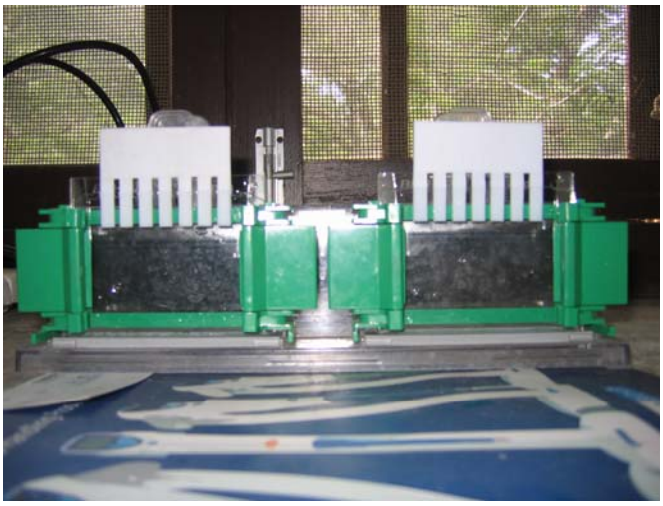


Figure 6.7. Loading the gels

Figure 6.8.

Figure 6.8. Loading the gels.

Figure 6.9.

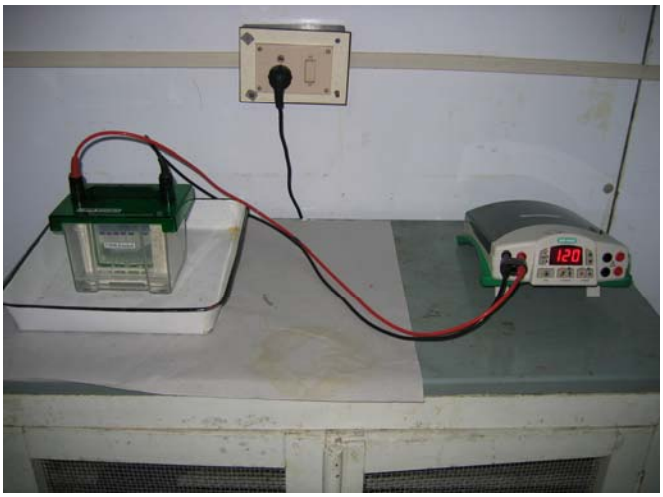


Figure 6.9. Electrophoresis

Figure 6.10.

Figure 6.10. Staining

Figure 6.11.

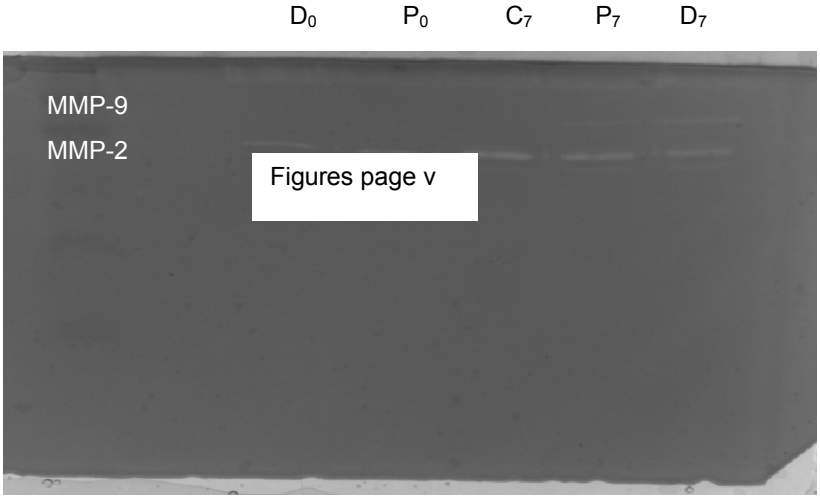


Figure 11. Zymogram after saline.
Expression of MMP-2 and MMP-9 is seen.

Figure 6.12.

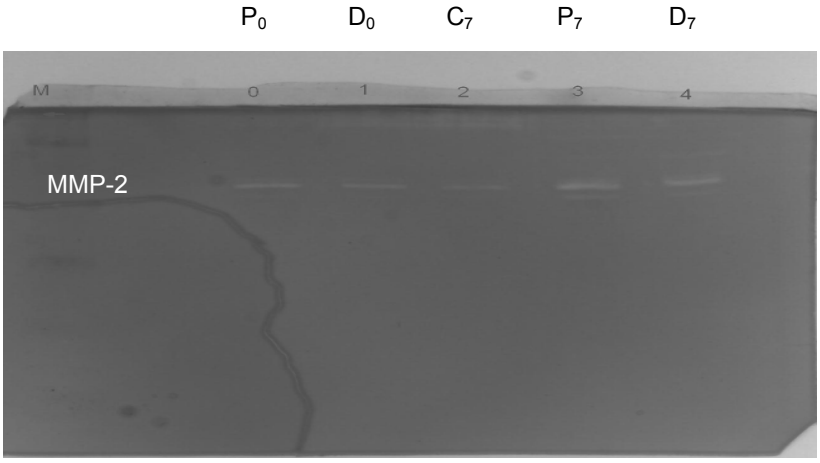
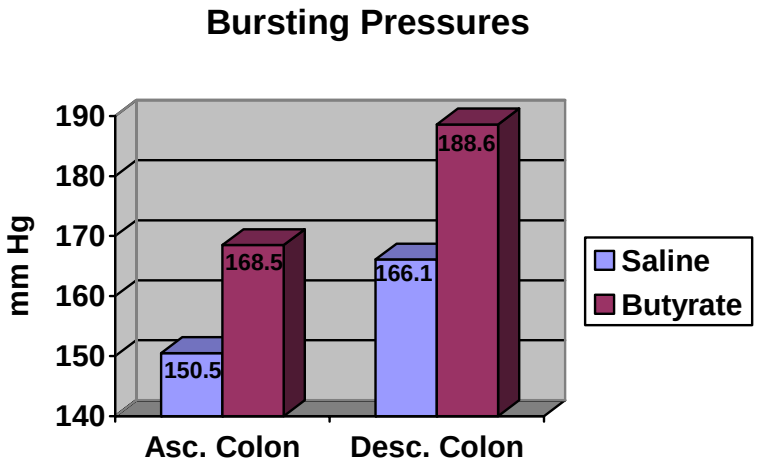


Figure 12. Zymogram after butyrate. Decreased expression of MMP-2 and absence of expression of MMP-9.

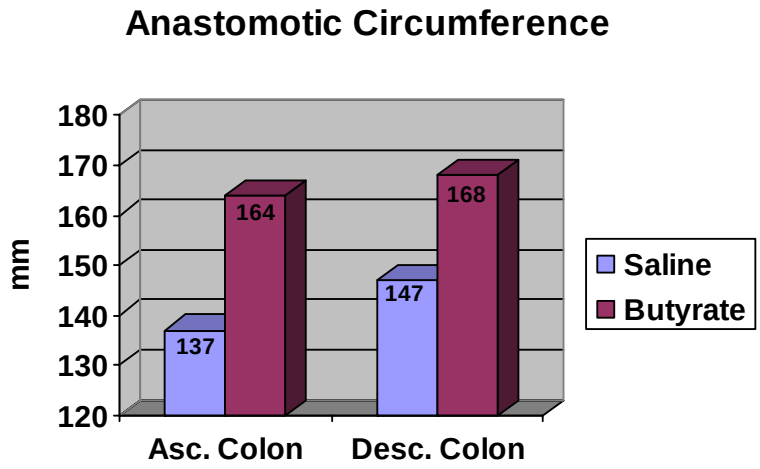
Legend
P_0 - Day 0. Proximal colon
D_0 - Day 0. Distal colon
C_7 - Day 7. Control
P_7 - Day 7. Proximal colon
D_7 – Day 7. Distal colon

7.0 Charts

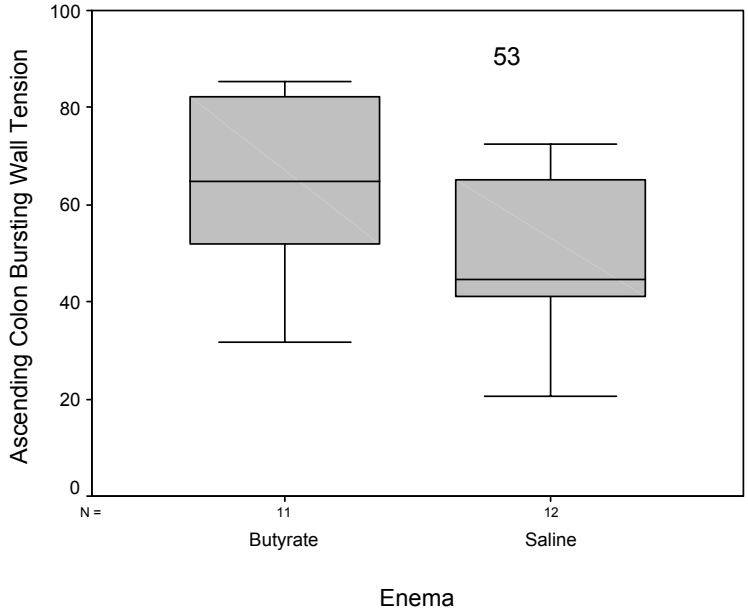
7.1. Bursting pressures with Saline and Butyrate



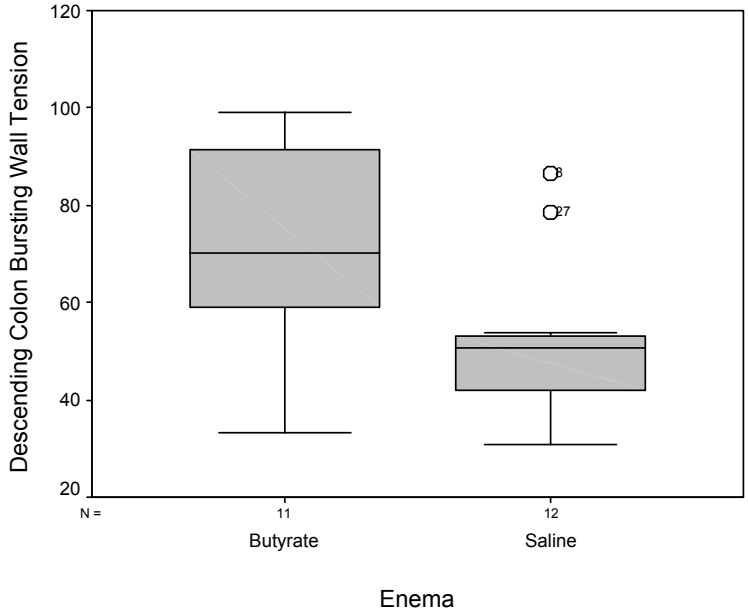
7.2 Anastomotic Circumferences with Saline & Butyrate



7.3. Ascending Colon Bursting Wall Tension



7.1.4 Descending Colon Bursting Wall Tension



8.0 Discussion

Colonic anastomoses have proved a challenge to surgeons for many decades due to the high rates of complications associated with them. Much work has gone into identifying the myriad factors associated with anastomotic healing and dehiscence in the colon. However, there still remains much room for experimentation before an ideal procedure can be identified and the rates of morbidity decreased.

This study was carried out to look at colonic anastomotic healing from a physiological perspective. With butyrate being a primary energy source of the colon, it was assumed that providing butyrate to a colon that was starved of it due to diversion would hasten healing. Preliminary work by other investigators in this field seemed to bear out this fact. This study has proved that providing intraluminal butyrate to a healing colon in the rat significantly strengthens the anastomosis and increases both the bursting pressure and the bowel wall tension.

The rat model was selected for this study as there was already some work in rats showing enhanced healing with butyrate.[91-95] The experimental model used was developed with some initial hiccoughs, especially with anaesthesia. Most other studies have used a single colonic anastomosis to study wound healing, usually in the right colon.[93-95] However, in this study, 2 anastomoses were used – in the

right and the left colon and it was found that this was a safe process, well tolerated by the animals.

The delivery of the butyrate was an area of struggle – with the rat as well as the method. There was no way of ensuring that the butyrate remained in the colon for sufficient time although considering the results, there was enough time for adequate action of the butyrate on the anastomoses. The other difficulty was that a few animals developed mucus or fecal plugs that prevented the butyrate from completely bathing the colon. In these animals, there was a corresponding drop in the bursting pressures and bowel wall tension.

The study shows that intraluminal butyrate strengthens colonic anastomoses both in the ascending and descending colon. Till date, most studies have dealt with the descending colon.[92-95] It was hypothesised that butyrate had a stronger action in the left colon. However, this study proves that there is a significant increase in the strength of even the ascending colonic anastomosis though the right colon anastomosis remains weaker than the left colon anastomosis in terms of bursting pressure and bowel wall tension. The bursting pressures and bowel wall tension of the right and left colon werehigher than those in some of the previouls studies as the rats used in those studies were of a different strain, namely Sprague-Dawley.

It was found that in a number of rats, the colon burst at a site away from the anastomosis. In all these rats, the site of bursting was within 1 cm from the anastomosis. This finding is in keeping with previous studies[111] and the possible explanation is that the zone of healing extends on either side of the anastomosis and in some instances, the colon is weakest at this point. This is because, the mechanical strength provided by the suture material, especially a non-absorbable one as in this case adds to the strength at the suture line when compared to a point just away from it.

An observation was made that there appeared to be more adhesions of the small bowel, omentum and seminal vesicles to the site of the anastomosis in the animals given saline enemas. As this was not part of the study protocol, there were no objective measurements of the adhesions that formed by the seventh post-operative day. However, towards the end of the study it appeared that animals given saline did have more adhesions than those given butyrate. While this may be an anecdotal finding, there is possibly a scientific explanation. It has been suggested that butyrate cytoprotection of colonic epithelial cells may be mediated through inhibition of heat Shock Protein 70 (45).[112] The heat shock proteins or stress proteins are important in the cellular response to stress and in cellular homeostatic functions such as protein synthesis and protein transport across membranes. Emerging evidence

supports a role for HSP in the inflammatory response, suggesting participation in cytokine signal transduction and the control of cytokine gene expression. Butyrate increased colonic epithelial cell survival in the face of lethal heat stress and significantly reduced HSP 70 expression in this study by Venkatraman et al. This indicates that butyrate plays a role in healing by reducing the inflammatory reponse. The reduction of inflammation would explain the decreased adhesions in the rats given butyrate when compared to the rats given saline.

The expression of Matrix Metalloproteinases (MMPs) was increased following anastomosis. There was an increase in both rats treated with butyrate and treated with saline. There was no increase in MMP2, but in MMP 9 there was a obvious increase in expression in the rats treated with saline following operation. This was an observational finding based on the clearance of the gelatine. Quantitative analysis of the proteins could not be carried out.

This finding needs to be studied further. It was planned to run reverse zymography gels to ascertain the expression of tissue inhibitors of metalloproteinase (TIMPs). However, due to technical difficulties in procuring the pure MMPs required to run the gel, this part of the study was unsuccessful. The small amounts of MMPs available commercially at high prices were not sufficient to impregnate an entire gel in sufficient quantity to view activity of TIMPs. If there was an increased

activity of TIMPs as well, this would suggest a rapid rate of collagen based remodelling which would explain the strengthening of the anastomoses by butyrate. Further study of TIMPs is planned once MMPs can be easily synthesised in the lab. This is being planned using human fibroblast cell line which expresses high amounts of MMPs.

The expression of MMP 9 was increased in the rats treated with saline. This finding would suggest a role of butyrate in decreasing the collagen breakdown by MMP 9, leading to enhanced healing. However, in the light of the increased expression of MMP 2 in the butyrate treated rats, this finding needs to be viewed with caution and further studies are required.

Chowcat et al investigated the expression of collagenases and tissue inhibitor of metalloproteinases (TIMPs) using antibodies to these proteins at the site of colonic anastomoses.[117] They found that the expression of MMPs and TIMPs was limited to the suture line. However, in this study, it was found that there was increased expression of MMPs even at a site away from the anastomosis when compared to the pre-operative expression. This would suggest that that trauma to the colon leads to increased synthesis of MMPs throughout the colonic mucosa with increased concentration at the site of the anastomosis.

8.0 Conclusions

i. Local instillation of butyrate significantly strengthens colonic anastomoses as measured by the bursting wall tension.

ii. The effect of butyrate increases both the bursting pressure and the circumference of the anastomosis.

iii. There is a difference in the strength of proximal and distal colonic anastomoses.

iv. There was an increase in the expression of Matrix metalloproteinases in all the post operative specimens. There was no difference in the expression of MMP2, but an increase in the expression of MMP9 between the butyrate and saline arms.

9.0 Clinical Significance and Need of Further Research

i. The fact that butyrate strengthens colonic anastomoses leads to many possibilities in clinical practice. The main concern needs to be an ideal drug delivery system that would deliver the butyrate intraluminally to the colon in the post-operative period.

ii. The finding that MMP-9 shows diminished secretion followed by treatment with butyrate suggests that there may be a role for MMP inhibitors to be used in the post-operative period to strengthen colonic anastomoses. However, this needs much further work before it can be conclusively justified.

iii. The role of inflammatory mediators like HSP and cytokines needs to be evaluated with regard to butyrate, especially as there appeared to be reduced inflammatory changes in the animals treated with butyrate.

iv. The ideal study would be a time-based study where animals were sacrificed and tested at different times in the healing process, say at days 2,4,6,8 and 10 and then at 2 weeks and 4 weeks after the initial operation. This would give us some idea

about the exact step and the time in wound healing sequence of events that is affected by butyrate.

v. There would need to be a multi-factorial approach to the experimental testing of the effect of butyrate on wound healing. This would allow the factors involved to be detected early and treatment mechanisms to be tested based on the factors implicated.

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Appendix 1

The Effect of Butyrate in the Healing of Colonic Anastomoses in Rats.

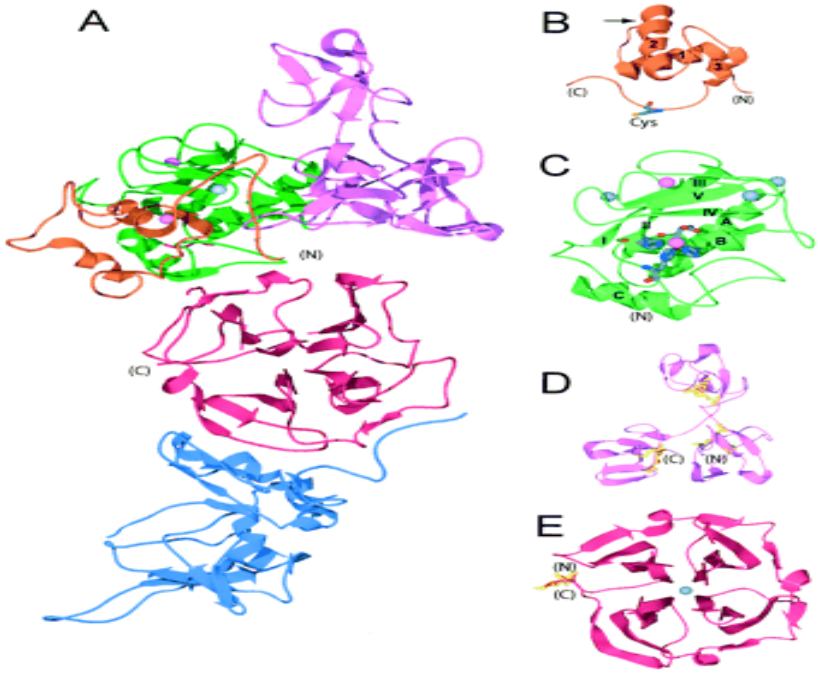
Spread Sheet

Rat Number	Enema	Proximal Colon Bursting Pressure	Distal colon Bursting Pressure	Proximal Colon Circum-ference	Distal colon Circum-ference	Proximal Colon Bursting Wall Tension	Distal colon Bursting Wall Tension
3	S	84	175	-	-	-	-
4	B	106	194	-	-	-	-
6	B	110	188	-	-	-	-
7	S	88	168	-	-	-	-
10	S	118	122	1.6	1.7	40.88	44.91
11	B	196	208	2	2.2	84.89	99.09
12	S	160	100	1.2	1.5	41.57	32.48
16	S	116	174	1.2	2.3	30.14	86.66
17	B	122	148	1.2	2	31.7	64.1
22	B	204	212	2	1.8	83.94	85.76
23	S	206	110	1.5	1.8	71.43	42.87
24	B	156	188	1.2	1.3	57.43	89.56
25	B	188	208	2	2.2	85.5	99.09
27	B	196	172	1.9	2.5	80.65	93.12
28	S	147	191	1.3	1.3	44.56	53.77
29	S	137	141	1.5	1.7	44.5	51.91
30	S	148	162	1.2	1.5	41.67	52.62
31	B	176	180	1.7	1.8	64.79	70.16
32	S	151	202	1.5	1.2	49.05	52.49
33	B	141	170	2	0.9	45.8	33.13
34	S	250	191	1.3	1.2	70.38	49.64
35	B	165	170	1.3	1.6	46.45	58.9
36	S	105	177	0.9	0.8	20.46	30.66
37	B	191	210	1.4	1.3	57.9	59.12
38	S	230	171	1.3	1.1	59.77	40.73
39	B	240	204	1.4	0.9	72.76	44.17
40	S	167	242	1.9	1.5	72.33	78.61

S = Saline B = Butyrate

Appendix 2.

Three Dimensional Structure of Metalloproteinases



Appendix 2. Ribbon Diagram of MMP Structures.

Three dimensional (3D) structure of matrixins showing the ribbon diagram of MMP structures. A, ProMMP-2–TIMP-2 complex (1GXD) is shown. Orange indicates propeptide; green, catalytic domain; pink, fibronectin domains; red, hemopexin domain; and blue, TIMP-2. Zinc atoms are pink, and calcium atoms are gray. B, In the MMP-2 propeptide, the cysteine of the cysteine switch motif is shown. The arrow indicates the position of the initial cleavage resulting in partial activation. C, The catalytic domain of MMP-1 is shown.

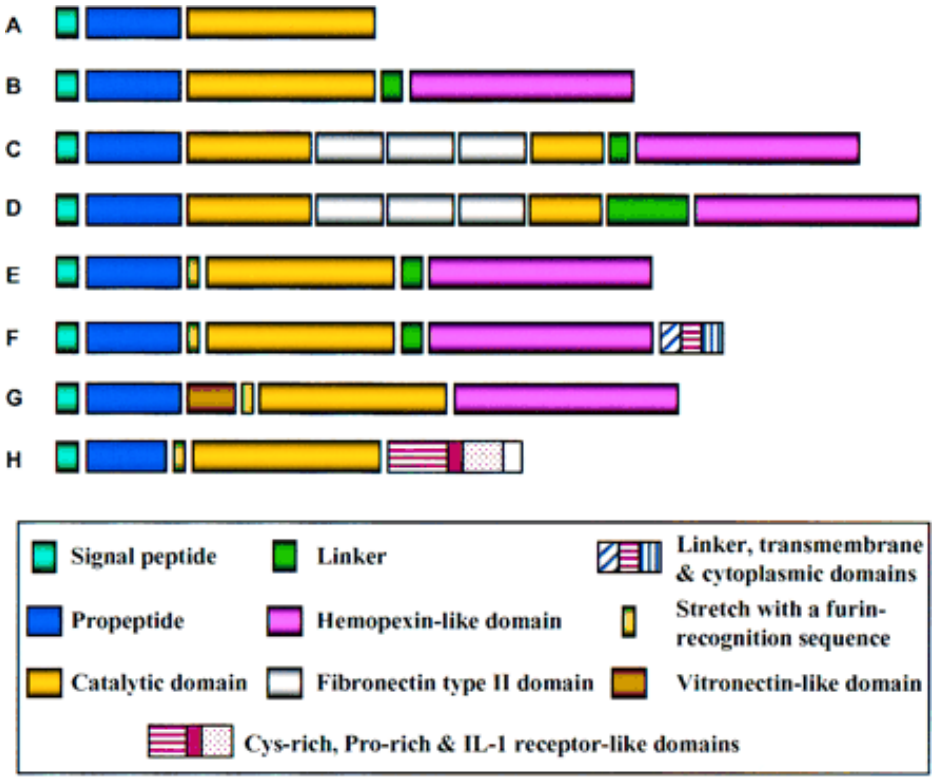
The ß-strands are numbered I through V; the α-helices are labeled A through C. The N-terminal (N) to C-terminal (C) order of the ß-stands and α-helices is I-A-II-III-IV-V-B-C. The histidines coordinating the active-site zinc and the active-site glutamic acid are shown. D, The 3 fibronectin domains of MMP-2^{40} are shown with their 2 disulfide bonds each. E, The hemopexin domain of MMP-1^{128} with 4 ß-propeller blades is shown. A disulfide bond is seen between blades I and IV.

This figure was prepared with a Swiss PDB Viewer and rendered with POV-Ray.

Visse R, Nagase H. Matrix Metalloproteinases and Tissue Inhibitors of Metallo-proteinases. Structure, Function, and Biochemistry Circulation Research. 2003; 92:827-39.

Appendix 3.

Domain Arrangements of Vertebrate Matrixins



Appendix 3. Domain Arrangements of Vertebrate Matrixins

The propeptide domain (about 80 amino acids) has a conserved unique sequence.

The catalytic domain (about 170 amino acids) contains a zinc binding motif HE*XX*H*XX*G*XX*H and a conserved methionine, which forms a unique "Met-turn" structure. This domain consists of a five-stranded β-sheet, three α-helices, and bridging loops. The catalytic domains of matrixins have an additional structural zinc ion and 2-3 calcium ions, which are required for the stability and the expression of enzymic activity. MMP-2 and MMP-9 have three repeats of fibronectin-type II domain inserted in the catalytic domain.

The C-terminal hemopexin-like domain (about 210 amino acids) has an ellipsoidal disk shape with a four bladed β-propeller structure; each blade consists of four antiparallel β-strands and an α-helix.

Nagase H, Woessner JF Jr. Matrix Metalloproteinases. J Biol Chem. 1999; Jul 30; 274(31):21491-4.

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